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T H E P A T E N T S A C T , 1970.

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It is hereby Certified that annexed hereto is a true copy of the Application on Form 1A, Complete Specification and Drawings filed in connection with Patent Application No.1239/MAS/98 dated the 9th June, 1998 by M/s. ASTRA AB, A SWEDISH COMPANY, AT S-151 85, SODERTALJE, SWEDEN.

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FORM 1A

APPLICATION FOR PATENT

By the Assignee or legal Representative of the True
and First Inventor.
(See Section 7)

(To be made in triplicate and shall be accompanied by three copies of the
provisional specification in Form J, or the complete specification in Form JA)

M/W/M/S: ASTRA AKTIEBOLAG, A SWEDISH COMPANY, AT S-151 85,
SODERTALJE, SWEDEN.

hereby declare:-

(i) that I am/we are in possession of an invention for
'NEW ASSAY KIT'
(ii) that I am/we/the said claim(s) to be the assignee(s) of or the legal representative(s) of

SEE ANNEXURE A ENCLOSED

who claim(s) and is/are believed to be the true and first inventor(s) thereof;

(iii) that the provisional/complete specification filed with this application is (and the complete specification) and any appended specification which may hereafter be filed in this behalf will be true of the invention to which this application relates;

(iv) that I/we believe that I am/we are entitled to a patent for the said invention having regard to the provisions of the Patents Act, 1970;

(v) that to the best of my/our knowledge, information and belief, the facts and matters stated herein are correct and that there is no lawful ground of objection to the grant of patent to me/us on this application. I/we request that a patent may be granted to me/us for the said invention.

I/we request that all notices, requisitions and communications relating to this application may be sent to MRS.A.V. NATHAN, at 451, 2nd Cross, 3rd Block, 3rd Stage, Basaveshwaranagar, BANGALORE-560 079, KARNATAKA STATE, INDIA.

Dated this 4TH day of JUNE, 1998

Nathanson

(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

To
The Controller of Patents,
The Patents office,
MADRAS.

ANNEXURE A

MR. T.S. BALGANESH,
NO. 47, 'SOUNDARYA' 5TH MAIN,
POSTAL COLONY, II STAGE,
SANJAYANAGAR,
BANGALORE - 560 094.

MR. UMENDER K. SHARMA,
NO. 385, 12TH CROSS,
VYALIKAVAL,
BANGALORE - 560 003.

MS. VASANTHI RAMACHANDRAN,
NO. 227, 11TH CROSS, II A MAIN,
1ST BLOCK, BEL LAYOUT,
VIDYARANYAPURA,
BANGALORE - 560 097.

FORM 3 A

THE PATENTS ACT, 1970
COMPLETE SPECIFICATION
(SEE SECTION-10)

'NEW ASSAY KIT'

WE, M/B. ASTRA AB, A SWEDISH COMPANY, AT S-151 85, SODERTALJE,
SWEDEN.

THE FOLLOWING SPECIFICATION PARTICULARLY DESCRIBES AND ASCERTAINS
THE NATURE OF THIS INVENTION AND THE MANNER IN WHICH IT IS TO BE
PERFORMED.

NEW ASSAY KIT

TECHNICAL FIELD

5 The present invention relates to a method of identifying a ligand, in particular an inhibitor, of a bacterial RNA polymerase sigma subunit.

BACKGROUND ART

10

Sigma subunits of RNA polymerase

Transcription of genes to the corresponding RNA molecules is a complex process which is catalyzed by DNA dependent RNA polymerase, and involves many 15 different protein factors. In eubacteria, the core RNA polymerase is composed of α , β , and β' subunits in the ratio 2:1:1. To direct RNA polymerase to promoters of specific genes to be transcribed, bacteria produce a variety of proteins, known as sigma (σ) factors, which interact with RNA polymerase to form an active holoenzyme. The resulting complexes are able to recognize and attach to selected 20 nucleotide sequences in promoters.

Physical measurements have shown that the sigma subunit induces 25 conformational transition upon binding to core RNA polymerase. Binding of the sigma subunit to the core enzyme increases the binding constant of the core enzyme for DNA by several orders of magnitude (Chamberlin, M.J. (1974) Ann. Rev. Biochem. 43, 721-).

Bacterial sigma factors do not have any homology with eukaryotic transcription factors, and are consequently a potential target for antibacterial compounds.

Mutations in the sigma subunit, effecting its association and ability to confer DNA sequence specificity to the enzyme, are known to be lethal to the cell.

Characterization of sigma subunits, identified and sequenced from various organisms, allows them to be classified into three groups. The Group I sigma has also been referred to the sigma⁷⁰ class, or the "house keeping" sigma group (for a review see Lonetto et al. (1992) J. Bacteriol. 174, 3843-3849). Sigma subunits belonging to this group recognize similar promoter sequences in the cell. These properties are reflected in certain regions of the proteins which are highly conserved between species.

Another important feature of sigma-dependent transcription is that the sigma subunit dissociates from the core enzyme during elongation of mRNA. Consequently, molecules which stabilize the interaction of the sigma subunit with the core enzyme would also be capable of inactivating transcription.

Mycobacterium tuberculosis

Mycobacterium tuberculosis is a major pulmonary pathogen which is characterized by its very slow growth rate. As a pathogen it gains access to the alveolar macrophages where it multiplies within the phagosome, finally lysing the cells and being disseminated through the blood stream, not only to other areas of the lung, but also to extrapulmonary tissues.

The pathogen thus multiplies in at least two entirely different environments, involving the utilization of different nutrients and a variety of possible host factors. A successful infection would thus involve the coordinated expression of new sets of genes, transcribed by RNA polymerases associating with different sigma factors. This opens the possibility of targeting not only the sigma⁷⁰ subunits

of *M. tuberculosis*, but also other sigma subunits specific for the different stages of infection and dissemination.

5 The cloning and expression of *Mycobacterium tuberculosis sigA* and *sigB* genes are disclosed in the International Patent Application WO 96/38478 (Astra AB).

Anti-sigma factors

Anti-sigma (Asi) proteins are known in the art. Lysates of bacteriophage T₂ (Khesin et al. (1972) Mol. Gen. Genet 119, 299) or phage T₄ (Bogdanova et al. (1970) Mol. Biol. 4, 435; Stevens, A. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 603) have been reported to inhibit transcription of bacterial genes.

It has been established that the T₄-dependent anti-sigma⁷⁰ activity is borne by a 10 kDa protein (Stevens, A. In: RNA Polymerase p. 617-627 (Eds. R. Losick & M. Chamberlin) Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1976). A 10 kDa protein was shown to co-purify with RNA polymerase from T₄ infected *E. coli* cells and is detached together with sigma⁷⁰ from the core enzyme on phosphocellulose columns.

20 A gene called *asiA*, coding for the 10 kDa anti-sigma⁷⁰ factor of bacteriophage T₄, has been identified by Orsini et al. (1993) J. Bacteriol. 175, 85-93. The open reading frame encoded a 90 amino acid protein with the deduced sequence

MNKNIDTVRE IITVASILIK FSREDIVENR ANFIAFLNEI GVTHEGRKLN

25 QNSFRKIVSE LTQEDKKTLI DEFNEGFEVG YRYLEMYTNK (SEQ ID NO: 5). The *asiA*-encoded protein was overproduced in a phage T₇ expression system and partially purified. It showed a strong inhibitory activity towards sigma⁷⁰-directed transcription by *E. coli* RNA polymerase holoenzyme. The nucleotide sequence of gene *asiA* has been deposited in the GenBank data base under accession no.

30 M99441.

Examples of proteins regulating the sigma subunit of RNA polymerase are known also from other systems. An example is the *S. typhimurium* flagellar regulation system, which is a complex system controlled by a set of over 50 genes grouped into 13 flagellar operons. Late operon expression is positively regulated by the *fliA* gene coding for the sigma factor FliA (Suzuki et al. (1978) J. Bacteriol. 133, 904; Suzuki et al. (1981) J. Bacteriol. 145, 1036). On the other hand, the late operons are negatively regulated by the *flgM* gene. A 7.8 kDa protein has been identified as the *flgM* gene product and purified (Ohnishi et al. (1992) Mol. Microbiol. 6, 3149-3157). This FlgM protein was identified as an anti-sigma factor since it was capable to bind the FliA protein and disturbed its ability to form a complex with the RNA polymerase core enzyme.

Similarly, in *B. subtilis* gene expression, the sigma^F factor has been shown to be regulated by a 14 kDa anti-sigma factor encoded by the *spolIAB* gene (Duncan & Losick (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2325-2329), while the sigma^B factor is regulated by a 16 kDa anti-sigma factor encoded by the *rsbW* gene (Benson & Haldenwang (1993) Proc. Natl. Acad. Sci. U.S.A. 90, 2330-2334).

The nucleotide sequences of the genes *flgM*, *spolIAB* and *rsbW* are available in the GenBank data base (Accession Nos.: FLGMST.PRO for *flgM*, SPOIIAB.PRO for *spolIAB*, M34995.PRO for *rsbW*). The sequences do not show any gross similarity with the *asiA* sequence disclosed by Orsini et al.

WO 96/25170 discloses methods for treating diseases caused by bacterial pathogens by administering the T₄ AsiA protein, which protein is shown to inhibit the RNA polymerase activity of *Bacillus subtilis* and *Mycobacterium smegmatis*.

DISCLOSURE OF THE INVENTION

In accordance with the present invention, there is provided a method of identifying a ligand of a bacterial sigma⁷⁰ subunit which comprises contacting the sigma⁷⁰ subunit or a portion thereof comprising the anti-sigma binding region, with a test compound and a fusion protein of an anti-sigma⁷⁰ factor of bacteriophage T₄, and determining whether the test compound binds competitively with the anti-sigma⁷⁰ factor to the sigma⁷⁰ subunit or portion thereof.

10

Preferably, the method comprises:

- (i) immobilizing the sigma⁷⁰ subunit or portion thereof on a matrix or solid support;
- (ii) adding the test compound and the fusion protein;
- 15 (iii) adding a first antibody against the fusion protein;
- (iv) adding a labeled second antibody against the first antibody; and
- (v) determining the amount of second antibody bound to the (first antibody - fusion protein - sigma⁷⁰ subunit or portion thereof) complex formed on the matrix or solid support.

20

The invention provides a method of screening peptide libraries or chemical libraries for compounds (ligands) which mimic the anti-sigma⁷⁰ factor and which thereby have the capability of inhibiting the interaction of core RNA polymerase and sigma⁷⁰ subunits. Such inhibitor compounds are potentially useful in the treatment of bacterial infections.

The capability of the identified ligands to inhibit the interaction between a bacterial core RNA polymerase and a sigma⁷⁰ subunit can be tested by known methods, in particular by a RNA polymerase assay, such as that disclosed in Orsini et al. (*supra*), wherein transcription of a DNA template, such as calf thymus DNA,

T₄ DNA or poly(dA-dT), is measured by the incorporation of a labeled RNA precursor such as [5-³H]UTP.

In the method according to the invention, the sigma⁷⁰ subunit or portion thereof is
5 preferably obtained from *Escherichia coli* (particularly the C-terminal 99 amino acids of the sigma⁷⁰ subunit containing the anti-sigma binding region) or *Salmonella typhimurium*.

In the fusion protein, the anti-sigma⁷⁰ factor of bacteriophage T₄ will preferably
10 have an amino acid sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 2.
The fusion protein preferably also comprises glutathione-S-transferase (GST-Asi)
or any other suitable marker gene protein product.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1: Map of plasmid vector pARC 8112.

Fig. 2: Map of plasmid vector pARC 8100.

20

Fig. 3: Map of plasmid vector pARC 8115.

Fig. 4: Map of plasmid vector pARC 8101.

25 Fig. 5: Map of plasmid vector pARC 8114.

Fig. 6: Map of plasmid vector pARC 8105.

Fig. 7: Map of plasmid vector pARC 8180.

30

Fig. 8: Activity of GST-Asi protein on sigma⁷⁰-dependent transcription.

Assay: 0.5 µg *E. coli* core polymerase, 2.5 µg sigma⁷⁰ protein, 1.0 µg T₄ DNA and NTPs. Varying concentrations of GST-Asi was preincubated with sigma protein before addition to the reaction mixture. ³H-UTP specific activity was 2800 - 3000 cpm/nmole.

EXAMPLES

Throughout this description the terms "standard protocols" and "standard procedures", when used in the context of molecular cloning techniques, are to be understood as protocols and procedures found in an ordinary laboratory manual such as: Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994, or Sambrook, J., Fritsch, E.F. and Maniatis, T., Molecular Cloning: A laboratory manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY 1989.

EXAMPLE 1: Cloning, expression and purification of *E. coli* sigma⁷⁰

PCR primers corresponding to the 5'- and 3'-ends of the coding sequences of *E. coli* sigma were designed with the 5'-sequence including a site for the restriction enzyme EcoRI and the 3'-sequence including a site for the restriction enzyme SalI. The PCR amplified DNA fragment was restricted with the above mentioned restriction enzymes and cloned into the EcoRI-SalI sites of the expression vector pTrc99a (Amann et al. (1988) Gene 69, 301), whereafter the ligation mix was transformed into *E. coli* DH5α. Transformants harboring the recombinant plasmid with the expected restriction profile were identified.

E. coli DH5 α cells harboring the recombinant plasmid, labeled pARC 8112 (Fig. 1), were grown at +37°C in LB till an OD (600 nm) of 0.4 and induced with 1 mM IPTG. More than 50% of the 90 kDa sigma⁷⁰ protein was found as inclusion bodies as had been reported earlier (Borukhov & Goldfarb (1993) Protein expression and purification 4, 503). The overexpressed sigma⁷⁰ was purified and renatured following standard protocols.

The activity of the purified sigma⁷⁰ protein was confirmed by its ability to support transcription mediated by *E. coli* RNA polymerase core enzyme. *E. coli* RNA polymerase core and holoenzyme forms were purified following the protocol of Burgess and Jendriask (1975) Biochemistry 14, 4634-4638. Polymerase activity using T₄ DNA as template was assayed as described by Orsini et al. (1993) J. Bacteriol. 175, 85-93.

15

EXAMPLE 2: Cloning, expression and purification of *Salmonella typhimurium* sigma⁷⁰

The coding sequence of the sigma⁷⁰ of *S. typhimurium* was amplified using the forward primer shown as SEQ ID NO: 3 and the reverse primer shown as SEQ ID NO: 4. The forward primer includes the site for the restriction enzyme EcoRI, while the reverse primer includes the sequence encoding the stop codon and a site for the restriction enzyme SalI. *S. typhimurium* DNA was used as template and the amplified fragment digested with appropriate enzymes. This fragment was then ligated to EcoRI - SalI digested pTrc 99A (Amann et al. (1988) Gene 69, 301) and transformed into *E. coli* DH5 α to obtain the recombinant plasmid pARC 8118. Plasmid DNA of pARC 8118 was digested with EcoRI - HindIII and the released 2.3 kb DNA fragment encompassing the entire coding sequence of *S. typhimurium* sigma⁷⁰ was cloned into the EcoRI - HindIII digested pRSET B (Kroll et al. (1993) DNA and Cell Biol. 12, 441) vector which has not only

the T₇ promoter but also sequences encoding a his tag which is now fused at the C-terminus of *S. typhimurium* sigma⁷⁰. The recombinant plasmid encoding the *S. typhimurium* sigma⁷⁰ fused to a His tag was labeled pARC 8133.

5 Plasmid DNA of pARC 8133 was then used to transform the expression host *E. coli* BL21(DE3) and transformants obtained at +37°C. Cells harboring pARC 8133 were grown in LB at +37°C till an OD of 0.4 at 600 nm and induced with 1 mM IPTG for 4 hours. The cells were pelleted, suspended in buffer and sonicated, according to standard methods. The lysed cells were clarified by centrifugation at 45,000 rpm
10 for 60 min at +4°C and the clarified supernatant was loaded directly on Ni-agarose (Pharmacia) and washed with 50 mM imidazole. The bound protein was eluted with 200 mM imidazole and dialysed overnight at +4°C against 50 mM Tris-Cl, pH 7.5. The eluate was concentrated using Amicon cone filters to 1/10 volume. The concentrated protein was cleaved with enterokinase, used at a ratio of 1:50 of
15 the enzyme, at +25°C for 14 hours. The cleaved mixture was passed through a Ni²⁺ agarose column and the unbound material collected. The cleaved protein was analyzed for homogeneity by SDS-PAGE.

20 EXAMPLE 3: Toxicity of the anti-sigma protein

3.1. *Cloning of the asiA gene*

25 The coding sequence for the *asiA* gene (Orsini et al., *supra*) was amplified from the genomic DNA of bacteriophage T₄ by PCR using the forward primer shown as SEQ ID NO: 5 and the reverse primer designated shown as SEQ ID NO: 6.

30 The 5'-primer included the sequence of the restriction enzyme *Nco*I while the 3'-primer included the sequence for the restriction enzyme *Bam*HI. The coding sequence was amplified by PCR following standard protocols and the amplified

fragment ligated to *NcoI* - *BamHI* restricted pBR329 and transformed into competent cells *E. coli* DH5 α . Recombinants were selected at +37°C as chloramphenicol sensitive, ampicillin resistant transformants. One of the transformants having the desired restriction pattern was labeled pARC 8100 (Fig. 2).

The inclusion of the sequence encoding *NcoI* resulted in the change of the second amino acid from asparagine to glycine (SEQ ID NO: 2). The nucleotide sequence of the *asiA* gene cloned in pARC 8100 was verified by double stranded sequencing and found to be identical to the *asiA* sequence as disclosed by Orsini et al., except

for the expected change of codon for the second amino acid as a result of the PCR cloning protocol used.

3.2. *In vivo* toxicity of *asiA* product in *E. coli*

15

(A)

The *NcoI* - *BamHI* DNA fragment from pARC 8100 was ligated to pET 8c Km (Umender K. Sharma et al., J. Bact., 177, 6745 (1996)) and kanamycin resistant transformants with *E. coli* DH5 α were selected at +37°C. One of the transformants harboring a plasmid with the expected restriction enzyme profile was labeled pARC 8115 (Fig. 3). pARC 8115 plasmid DNA was then used to transform the expression host *E. coli* BL21(DE3) (Studier et al., *supra*) and transformants selected both at +37°C and at +30°C. However, no viable transformants could be obtained at either of the temperatures. The leaky expression from the T7 promoter in pET 8c being much higher than from pET 11d(Km) (Studier et al., *supra*), the toxicity of the *asiA* product could explain the non-transformability.

Transformants could however be obtained using *E. coli* BL21(DE3)/pLysS as host, in which the leaky expression is additionally repressed by the T7 lysozyme expressed from pLysS.

(B)

The *NcoI - BamHI* 284 bp DNA fragment was obtained from pARC 8100 and ligated to the *NcoI - BamHI* sites of pET 11d Km (Umender K. Sharma et al., J. Bact., 177, 6745 (1996)) and transformed into *E. coli* DH5 α . Transformants were selected for kanamycin resistance. Plasmid preparations from individual transformants were digested with restriction enzymes and the correct transformant that released the 284 bp fragment after *NcoI - BamHI* digest was labeled pARC 8101 (Fig. 4). The transformants were selected at +37°C and appeared normal.

10

pARC 8101 DNA was then used to transform the expression host *E. coli* BL26(DE3). BL26 is a lac Iq isogenic strain of BL21 (Studier et al., *supra*). Transformants selected for kanamycin both at +37°C and at +30°C. The transformants obtained at +37°C were morphologically sick and non-viable indicating the acute toxicity of the leaky expression of the *asiA* gene. In contrast, healthy colonies could be obtained when transformants were selected at +30°C where the leaky expression from the T7 promoter can be expected to be negligible.

BL26(DE3)/pLysS colonies were healthy both at +37°C and +30°C, indicating that the tight regulation of expression in this strain made the *asiA* gene non-toxic to the host. Transformants obtained from DH5 α were also healthy both at +37°C and +30°C, indicating that the gene when transformed into a non-expression host was non-toxic.

25

(C)

The *asiA* gene was excised from pARC 8101 as a *XbaI - BamHI* DNA fragment to include the sequence for the ribosome binding site and ligated to the low copy vector, *XbaI - BamHI* cleaved pWKS129 (Wang & Kushner (1991) Gene 100, 195) and the ligation mix transformed to *E. coli* DH5 α . Recombinant plasmid harboring

the *asiA* gene was identified by restriction profile and labeled pARC 8114 (Fig. 5). This plasmid DNA when transformed into *E. coli* BL21(DE3) gave viable transformants both at +37°C and +30°C, since the low copy number of the plasmid reduced the level of the AsiA protein expressed through leaky expression.

5

EXAMPLE 4: Glutathione-S-transferase - Anti-sigma fusion protein

4.1. Cloning of fusion protein in *E. coli*

10

The coding sequence for the *asiA* gene was excised as an *NcoI* - *BamHI* fragment from pARC 8100 and ligated to *NcoI* - *BamHI* cleaved pARC 0499. The plasmid pARC 0499 has a *NcoI* site in frame with the glutathione-S-transferase encoding sequence, enabling fusion of the N-terminus of *asiA* sequences. The ligation mix was transformed into *E. coli* DH5 α and transformants selected at +37°C and +30°C. Viable colonies were obtained at both temperatures indicating the N-terminal fusion reduces the toxicity of the protein. The recombinant plasmid obtained with the sequences encoding GST-AsiA was labeled pARC 8105 (Fig. 6).

15

The GST-AsiA fusion protein was purified as follows: *E. coli* DH5 α harboring pARC 8105 was grown in LB till an OD at 600 nm of 0.6 at +37°C followed by the addition of 1 mM IPTG and allowing growth for a further period of 2 hours. The cells were then centrifuged at 5000 rpm for 10 minutes. The pelleted cells were suspended in Buffer A (phosphate buffered saline (PBS) pH 7.5, 5 μ g/ml aprotinin, 25 5 μ g/ml leupeptin) and sonicated. The sonicate was clarified at 45,000 rpm for 10 minutes at +4°C and the supernatant passed through a Glutathione-Sepharose 4B column previously equilibrated with PBS. The column was washed with 5 bed volumes of 1 M urea once followed by washing with PBS (10 bed volumes). The bound protein was eluted with 10 mM glutathione and the eluate dialysed

overnight against buffer containing 50 mM Tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl), pH 7.5. The dialysed protein was then concentrated to 1/10 vol using Amicon cone filters. The concentrated protein was then treated with 1:50 ratio of Factor Xa protease in a buffer containing 50 mM Tris HCl, pH 7.5, 100 mM NaCl, 1 mM CaCl₂ at +25°C for 4 hours. The digested eluate was then passed through a Glutathione-Sepharose column and the unbound fraction collected and analyzed for the purity of the AsiA protein by SDS-PAGE.

4.2. Cloning of fusion protein in yeast

The expression and purification of the GST-AsiA fusion protein in *E. coli* did not allow large scale production because of the inherent toxicity of the fusion protein. To obtain large quantities of the AsiA protein, the possibility of expressing the GST-AsiA fusion protein in a yeast host was investigated. As the *Saccharomyces* species do not have a sigma⁷⁰ homologue, the AsiA protein was expected not to have toxicity effects on the transcription apparatus of the host and thus for initial experiments a *Saccharomyces cerevisiae* expression system was chosen.

The gene encoding the GST-AsiA fusion protein on the plasmid pARC 8105 was amplified using the forward primer shown as SEQ ID NO: 7 and the reverse primer shown as SEQ ID NO: 8. The amplified 1.0 kb fragment was restricted with *Bgl*II and *Hind*III which are the sites introduced by the primer sequence at the 5'- and 3'-ends of the amplified PCR fragment. The *Bgl*II – *Hind*III restricted fragment was ligated into the *Bgl*II – *Hind*III restricted *Saccharomyces cerevisiae* cloning vector pSW6 (Pascall et al. (1991) J. Mol. Endocrinol. 6, 63-70). The ligation mixture was transformed into the *Saccharomyces cerevisiae* host CGY1585 and transformants selected on leucine (3 µg/ml).

Individual transformants from both reaction mixtures were screened for the presence of plasmid with the expected restriction profile. The recombinant

plasmid obtained with the vector PSW6 was labeled pARC 8180 (Fig. 7).

S. cerevisiae CGY 1585 strain harbouring pARC 8180 were grown using standard procedures and lysed by passing the concentrated cell suspension through a French Press. The lysate was then centrifuged at 10,000 rpm and the supernatant applied to a Glutathione-Sepharose column (Pharmacia) and the fusion protein purified following standard protocols.

The purified GST-AsiA fusion protein was cleaved with Factor Xa as recommended and the AsiA protein separated. The yield of the purified AsiA protein was 500 µg/l. The activity of the GST-AsiA protein, expressed and purified from *Saccharomyces*, was compared to that obtained from *E. coli* and found to be identical in its ability to inhibit sigma⁷⁰ mediated transcription of T₄ template (Fig. 8). AsiA protein purified from GST-AsiA after Factor Xa cleavage also inhibited *E. coli* sigma⁷⁰ dependent transcription.

15

The ability to overexpress and purify large quantities of the AsiA protein in *Saccharomyces* without toxicity problems for the host also indicates that the AsiA protein does not have a corresponding sigma⁷⁰ homologue in this species. As the *Saccharomyces* RNA polymerase is similar to that of higher eukaryotes it also substantiates the fact that the AsiA protein is toxic only to the prokaryotic transcription apparatus.

In order to test the feasibility of expressing AsiA in alternative yeast expression systems, *Pichia pastoris* expression system was chosen. The gene encoding GST-AsiA was amplified by PCR from pARC 8105 and cloned into pPIC9K (Invitrogen).

5' primer (asi34) - TA TAC GTA TCC CCT ATA CTA GGT TAT TGG

3' primer (asi35) - T TGC GGC CGC TTA TTT GTT CGT ATA CAT

30

The purified GST-AsiA fusion protein was cleaved with Factor Xa as recommended and the AsiA protein separated. The

PCR conditions used were:

melting temperature	94 °C
annealing temperature	50 °C
extension temperature	72 °C

and 30 cycles were performed.

5 1Kb PCR product was cloned as SnaBI-NotI fragment into pPIC9K (pARC 8274). *Pichia pastoris* transformants of pARC 8274 were selected based on G 418 resistance. Expression studies indicated that GST-AsiA was secreted into the culture supernatant. GST-AsiA purified from the culture supernatant was found to
10 inhibit *in vitro* transcription as efficiently as that of either *E. coli* or the *Saccharomyces* GST-AsiA.

This indicates that it is feasible to produce recombinant GST-AsiA in a yeast expression system.

15 EXAMPLE 5: *In vitro* AsiA-sigma⁷⁰ interaction assays

5.1. RNA polymerase assay

20 The *E. coli* RNA polymerase assay was standardized following the protocol of Orsini et al. (J. Bacteriol. 175, 85-93, 1993) using T4 phage DNA as template to quantify sigma⁷⁰ dependent transcription. *E. coli* RNA polymerase core enzyme was purified following the protocol of Burgess and Jendriask (1975) Biochemistry
25 14, 4634-4638.

5.2. Inhibition of *E. coli* transcription by AsiA protein

Increasing concentrations of AsiA protein expressed in *S. cerevisiae* was added to the reaction mixture and the transcription mediated by the *E. coli* RNA polymerase quantified. The results showed that increasing concentrations of AsiA protein completely inhibited transcription mediated by *E. coli* sigma⁷⁰.

5.3. Inhibition of *E. coli* transcription by GST-AsiA fusion protein

The GST-AsiA fusion protein was purified from *S. cerevisiae* as described in Section 5.2 and used at different concentrations in the *E. coli* sigma⁷⁰ mediated RNA polymerase transcription assay, using phage T₄ DNA as template. The GST-AsiA fusion protein inhibited >80% the sigma⁷⁰ mediated transcription when the core enzyme was reconstituted with 2.5 µg of sigma⁷⁰ protein.

15

5.4. Reactivation of *E. coli* sigma⁷⁰ mediated transcription

Increasing concentrations of *E. coli* sigma⁷⁰ protein were added to the reaction mixture containing 0.1 µg of AsiA. As shown in Table 1, addition of sigma⁷⁰ protein could reactivate transcription mediated by *E. coli* RNA polymerase. This reversal of inhibition demonstrates the specific interaction of the AsiA protein with sigma⁷⁰.

TABLE 1

	<i>E. coli</i> sigma ⁷⁰	<i>S.</i> <i>typhimurium</i> sigma ⁷⁰
[³ H]UTP (nmol)		
<i>E. coli</i> RNA polymerase	2.1	2.3
<i>E. coli</i> RNA polymerase + 0.1 µg AsiA	0.57	0.34
<i>E. coli</i> RNA polymerase + 0.1 µg AsiA + 4 µg sigma ⁷⁰	2.3	3.0
<i>E. coli</i> RNA polymerase + 0.1 µg AsiA + 4 µg sigma ⁷⁰ (AsiA preincubated)	0.68	0.98

5.5. Effect of *Salmonella typhimurium* sigma⁷⁰ on *E. coli* transcription

5

As described in Section 5.1, the *E. coli* RNA polymerase activity assay was standardized with purified enzyme and phage T₄ DNA as template. The RNA polymerase could be >80% inhibited by 0.07 µg of purified AsiA protein.

10

To a reaction mixture containing the *E. coli* RNA polymerase, T₄ DNA and 0.1 µg of AsiA, increasing concentrations of purified *Salmonella typhimurium* sigma⁷⁰ was added and the RNA polymerase activity quantified. As shown in Table 1, addition of 4 µg of *S. typhimurium* sigma⁷⁰ could restore the activity of the RNA polymerase.

15

5.6. Preincubation of sigma⁷⁰ with AsiA

To a reaction mixture containing *E. coli* RNA polymerase and T₄ DNA template

there was added 0.1 µg AsiA and 4 µg *E. coli* or *S. typhimurium* sigma⁷⁰,

E. coli RNA polymerase

T₄ DNA polymerase

E. coli RNA polymerase + 0.1 µg AsiA

+ 4 µg sigma⁷⁰

preincubated at +37°C for 10 min with 0.1 µg of AsiA. As shown in Table 1, preincubation of the sigma⁷⁰ with AsiA abolished the ability of the sigma⁷⁰, from both *E. coli* and *S. typhimurium*, to activate *E. coli* RNA polymerase.

5

EXAMPLE 6: Competitive ELISA for quantification of AsiA-sigma⁷⁰ interaction

Screening assays

10 The above principles provide a basis for a method of identification of oligonucleotide sequences encoding peptides, which may be either related or unrelated to the AsiA peptide, and which could efficiently bind to sigma⁷⁰ subunits from *E. coli*, *S. typhimurium* and/or other housekeeping or virulence-associated sigma subunits of bacterial pathogens.

15

The identification of such peptide structures would enable the further identification, by known methods, of peptoids, peptidomimetics and organic molecules which can be tested in transcription assays for inhibition of sigma⁷⁰ dependent transcription.

20

Truncated sigma⁷⁰ (C-terminal 99 amino acids) which has been shown to be the anti-sigma binding region of sigma⁷⁰ of *E. coli* (Severinova et al., J. Mol. Biol. (1996) 263 (5), pp 637-647) was cloned and expressed in a His tag expression vector resulting in a (His)₅-tagged protein product.

25

A solution of the protein product (200 pmoles/ml) in a pH 7.5 buffer comprising Tris HCl (10 mM) and sodium chloride (50 mM) was prepared and 200 µl of solution were added per well of a nickel-coated microtitre plate, followed by incubation at room temperature for a period of two hours. The microtitre plate

30 was then washed 5 times with the buffer solution and then incubated at 37°C for

one hour with a solution of bovine serum albumin (3% w/v) in the above buffer. After washing 5 times with phosphate buffered saline (PBS) with "Tween" 20 (polyoxyethylene (20) sorbitan monolaurate, 0.5% v/v), the GST-AsiA fusion protein prepared as described in *Example 4.1.* above (100 ng) was added together with a defined amount of a putative inhibitor of truncated sigma⁷⁰ protein (corallopyronin, myxopyronin or ripostatin) and incubated at 37°C for one hour. Further washing (5 times) with PBS-"Tween" 20 was carried out and then anti-GST antibodies (1:2000) raised in rabbits were added to the wells, with incubation at 37°C for one hour. The previous washing step was repeated and thereafter anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (1:2000) was added and incubated at 37°C for one hour. A final wash with PBS-"Tween" 20 was carried out, before adding the HRP enzyme substrate, tetramethyl benzidine / H₂O₂. The enzymic reaction (resulting in the development of colour) was stopped after a suitable period of time by the addition of 6N H₂SO₄ and the microtitre plate was "read" in a spectrophotometer using light of wavelength (λ) 450 nm. The results obtained are shown in Table 2 following.

TABLE 2

Test	% Binding to truncated sigma ⁷⁰
GST-AsiA (500 ng/ml)	100
GST-AsiA + AsiA (7 µg/ml)	12
GST-AsiA + corallopyronin (30 µg/ml)	69
GST-AsiA + myxopyronin (30 µg/ml)	56
GST-AsiA + ripostatin (1 µg/ml)	100
GST-AsiA + ripostatin (10 µg/ml)	55
GST-AsiA + ripostatin (100 µg/ml) with a defined amount of a putative inhibitor of truncated sigma ⁷⁰	1.5

After washing 5 times with PBS-"Tween" 20 was carried out and then anti-GST antibodies (1:2000) raised in rabbits were added to the wells, with incubation at 37°C for one hour. The previous washing step was repeated and thereafter anti-rabbit IgG-horse radish peroxidase (HRP) conjugate (1:2000) was added and incubated at 37°C for one hour. A final wash with PBS-"Tween" 20 was carried out, before adding the HRP enzyme substrate, tetramethyl benzidine / H₂O₂. The enzymic reaction (resulting in the development of colour) was stopped after a suitable period of time by the addition of 6N H₂SO₄ and the microtitre plate was "read" in a spectrophotometer using light of wavelength (λ) 450 nm. The results obtained are shown in Table 2 following.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(A) NAME: Astra AB
 (B) STREET: Västra Mälarehamnen 9
 (C) CITY: Södertälje
 (E) COUNTRY: Sweden
 (F) POSTAL CODE (ZIP): S-151 85
 (G) TELEPHONE: +46-8-553 260 00
 (H) TELEFAX: +46-8-553 288 20
 (I) TELEX: 19237 astra s

(ii) TITLE OF INVENTION: RNA Polymerase Inhibitors

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
 (B) COMPUTER: IBM PC compatible
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 90 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS:
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Bacteriophage T4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

Met Asn Lys Asn Ile Asp Thr Val Arg Glu Ile Ile Thr Val Ala Ser
 1 5 10 15

Ile Leu Ile Lys Phe Ser Arg Glu Asp Ile Val Glu Asn Arg Ala Asn
 20 25 30

Phe Ile Ala Phe Leu Asn Glu Ile Gly Val Thr His Glu Gly Arg Lys
 35 40 45

Leu Asn Gln Asn Ser Phe Arg Lys Ile Val Ser Glu Leu Thr Gln Glu
 50 55 60

Asp Lys Lys Thr Leu Ile Asp Glu Phe Asn Glu Gly Phe Glu Gly Val
 65 70 75 80

Tyr Arg Tyr Leu Glu Met Tyr Thr Asn Lys
 85 90

(B) STREET: Västra Mälarehamnen 9
 (C) CITY: Södertälje
 (E) COUNTRY: Sweden
 (F) POSTAL CODE (ZIP): S-151 85
 (G) TELEPHONE: +46-8-553 260 00
 (H) TELEFAX: +46-8-553 288 20
 (I) TELEX: 19237 astra s

RNA Polymerase Inhibitors

PatentIn Release #1.0, Version #1.30

IBM PC compatible

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 90 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS:
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: Bacteriophage T4

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met	Gly	Lys	Asn	Ile	Asp	Thr	Val	Arg	Glu	Ile	Ile	Thr	Val	Ala	Ser
1															15

Ile	Leu	Ile	Lys	Phe	Ser	Arg	Glu	Asp	Ile	Val	Glu	Asn	Arg	Ala	Asn
															30

Phe	Ile	Ala	Phe	Leu	Asn	Glu	Ile	Gly	Val	Thr	His	Glu	Gly	Arg	Lys
															45

Leu	Asn	Gln	Asn	Ser	Phe	Arg	Lys	Ile	Gly	Val	Ser	Glu	Leu	Thr	Gln	Glu
																60

Asp	Lys	Lys	Thr	Leu	Ile	Asp	Glu	Phe	Asn	Glu	Gly	Phe	Glu	Gly	Val
															80

Tyr	Arg	Tyr	Leu	Glu	Met	Tyr	Thr	Asn	Lys						
															90

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGGAATTCA ACCCGCAGTC ACAGCTG

27

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "PCR primer"

ATGGAATTCA ACCCGCAGTC ACAGCTG

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TGAGTCGACT TAATCGTCGA GGAAGCT

27

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR prim

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

25

(2) INFORMATION FOR SEQ ID NO: 6:

(ii) MOLECULE TYPE: other nucleic acid
· (A) DESCRIPTION: /desc = "PCR

(i) SEQUENCE CHARACTERISTICS

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GGGGATCCTT ATTTGTTCGT ATACAT

26

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "PCR primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

-GAAAGATCTC -ATATGTCCCC TATACTAGGT

30

JOURNAL OF MATHEMATICAL ANALYSIS AND APPLICATIONS

1. A BAG CONTAINS 5 RED AND 4 GREEN APPLES.
(A) 2 RED APPLES
(B) 3 GREEN APPLES
(C) 5 RED APPLES
(D) 9 APPLES

(A) $\text{DFT}(\text{HOMO}) - \text{DFT}(\text{LUMO}) = 5.0$ eV

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CTAAGCTTTT ATTTGTTCGT ATACATCTC

29

C L A I M S

1. A method of identifying a ligand of a bacterial sigma⁷⁰ subunit which comprises contacting the sigma⁷⁰ subunit or a portion thereof comprising the anti-sigma binding region, with a test compound and a fusion protein of an anti-sigma⁷⁰ factor of bacteriophage T₄, and determining whether the test compound binds competitively with the anti-sigma⁷⁰ factor to the sigma⁷⁰ subunit or portion thereof.
- 10 2. A method according to claim 1, which comprises:
 - (i) immobilizing the sigma⁷⁰ subunit or portion thereof on a matrix or solid support;
 - (ii) adding the test compound and the fusion protein;
 - (iii) adding a first antibody against the fusion protein;
 - 15 (iv) adding a labeled second antibody against the first antibody; and
 - (v) determining the amount of second antibody bound to the (first antibody - fusion protein - sigma⁷⁰ subunit or portion thereof) complex formed on the matrix or solid support.
- 20 3. A method according to claim 1 or claim 2, wherein the sigma⁷⁰ subunit or portion thereof is obtained from *Escherichia coli* or *Salmonella typhimurium*.
4. A method according to any one of the preceding claims, wherein the anti-sigma⁷⁰ factor has an amino acid sequence as shown in SEQ ID NO: 1 or SEQ ID NO: 2.
- 25 5. A method according to any one of the preceding claims, wherein the fusion protein also comprises glutathione-S-transferase.

6. A method according to any one of the preceding claims wherein the ligand is an inhibitor of a bacterial sigma⁷⁰ subunit.
7. A kit for a method of identifying a ligand of a bacterial sigma⁷⁰ subunit which comprises contacting the sigma⁷⁰ subunit or a portion thereof comprising the anti-sigma binding region, with a test compound and a fusion protein of an anti-sigma⁷⁰ factor of bacteriophage T₄, and determining whether the test compound binds competitively with the anti-sigma⁷⁰ factor to the sigma⁷⁰ subunit or portion thereof.
8. A kit as in claim 7 comprising of a fusion protein of an Antisigma⁷⁰ factor of bacteriophage T₄, Bacterial sigma⁷⁰ subunit or a portion thereof comprising the anti sigma binding region, solid phase matrix suitable to bind proteins, antibody to a fusion protein of an Antisigma⁷⁰ factor of bacteriophage T₄, labeled second antibody against the first antibody to fusion protein of an Antisigma⁷⁰ factor of bacteriophage T₄ and a suitable reagent to determine the label on the second antibody.
9. A process of preparing a kit claimed in claim 7 comprising of steps for producing a fusion protein of an Antisigma⁷⁰ factor of bacteriophage T₄.
10. A process of preparing a kit claimed in claim 7 comprising of steps for producing a fusion protein of an Antisigma⁷⁰ factor of bacteriophage T₄ is a fusion of glutathione transferase fusion to antisigma factor of bacteriophage T₄.
11. A process of producing a fusion protein of an Antisigma⁷⁰ factor of bacteriophage T₄ with glutathione transferase using a recombinant yeast expression system.

DATED THIS 4TH DAY OF JUNE, 1998.

which comprises contacting the sigma⁷⁰ subunit or a portion thereof

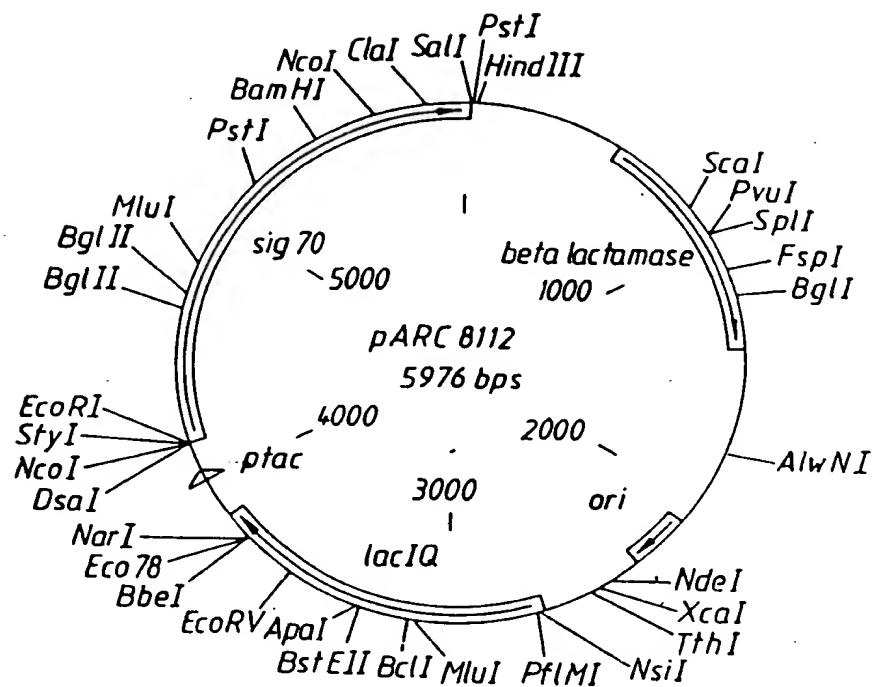
comprising the anti-sigma binding region, with a test compound and a fusion protein of an anti-sigma⁷⁰ factor of bacteriophage T₄, and determining whether the test compound binds competitively with the anti-sigma⁷⁰ factor to the sigma⁷⁰ subunit or portion thereof.

(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

ATTESTED AND SIGNED IN THE PRESENCE OF THE APPLICANT ON BEHALF OF THE APPLICANT.

ATTESTED AND SIGNED IN THE PRESENCE OF THE APPLICANT ON BEHALF OF THE APPLICANT.

ATTESTED AND SIGNED IN THE PRESENCE OF THE APPLICANT ON BEHALF OF THE APPLICANT.



A. V. Nathan
(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

F-7 9-1

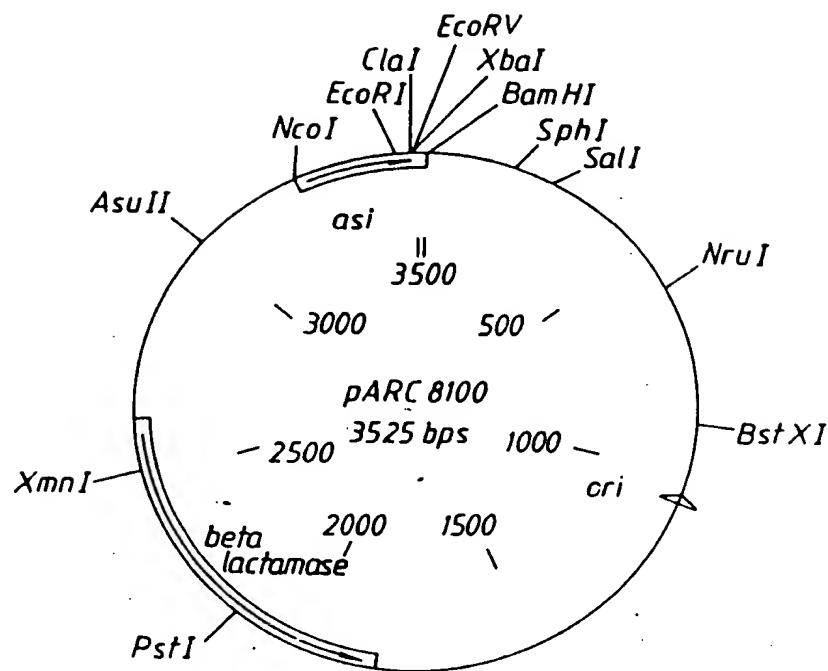


Fig. 2

A. Nathan
(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

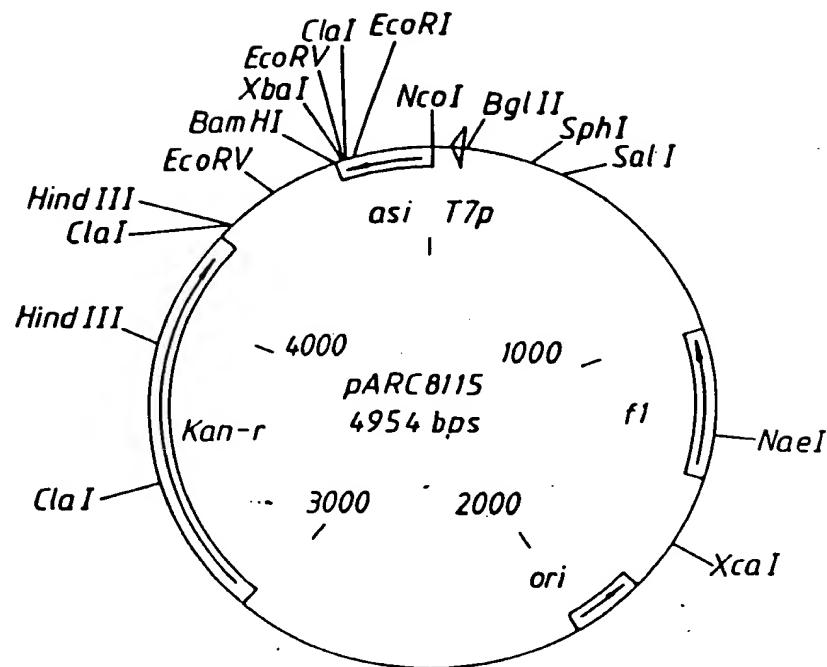


Fig. 3

Acvalton
(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

M/S. ASTRA AB
NO.

NO. OF SHEETS 8
SHEET NO. 4

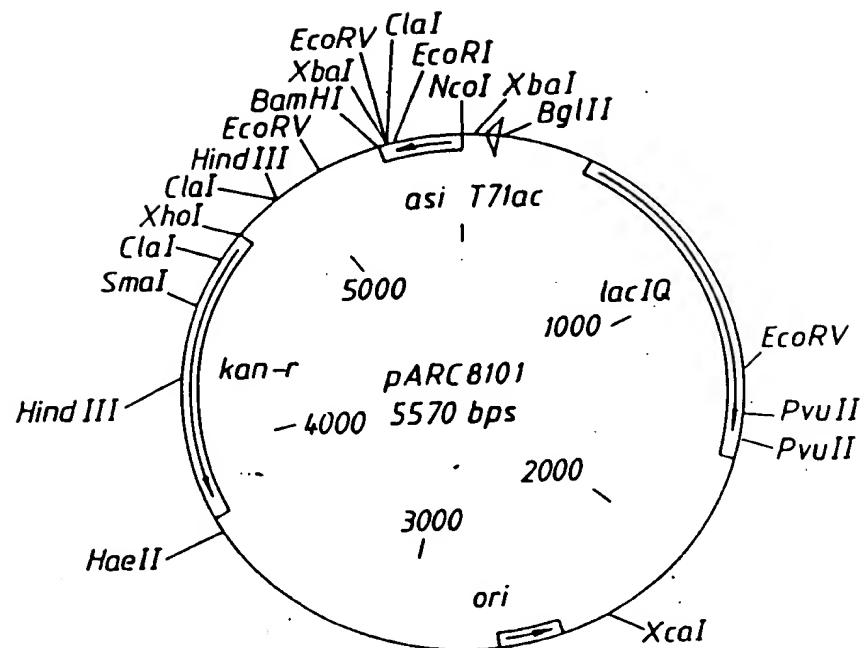


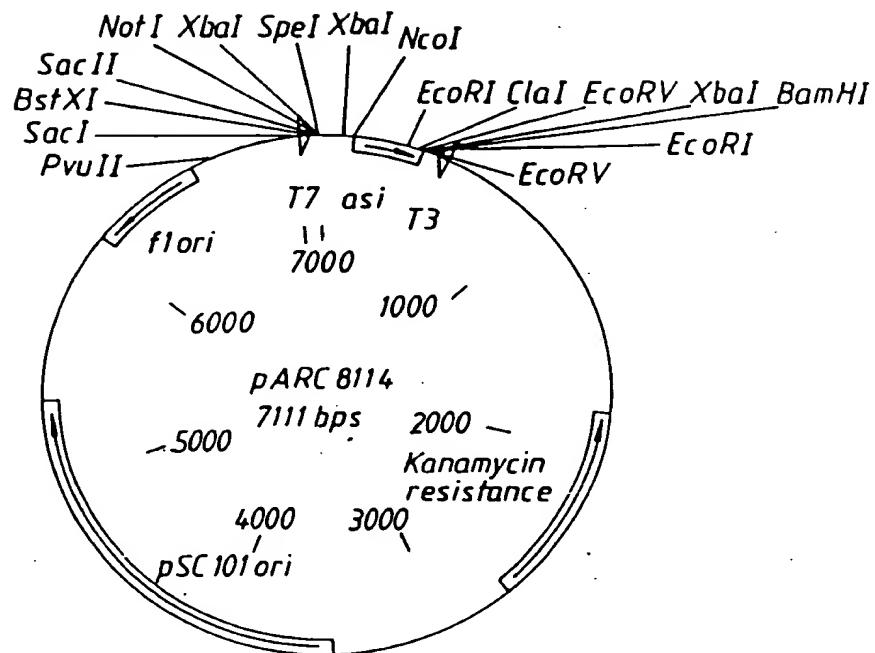
Fig. 4

Agralton

(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

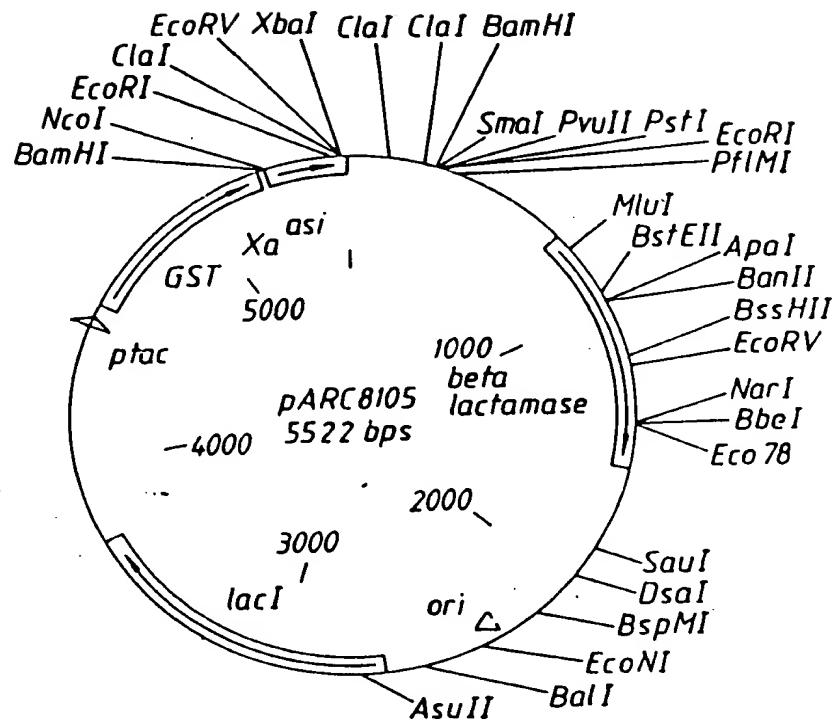
M/S. ASTRA AB
NO.

NO. OF SHEETS 8
SHEET NO. 5



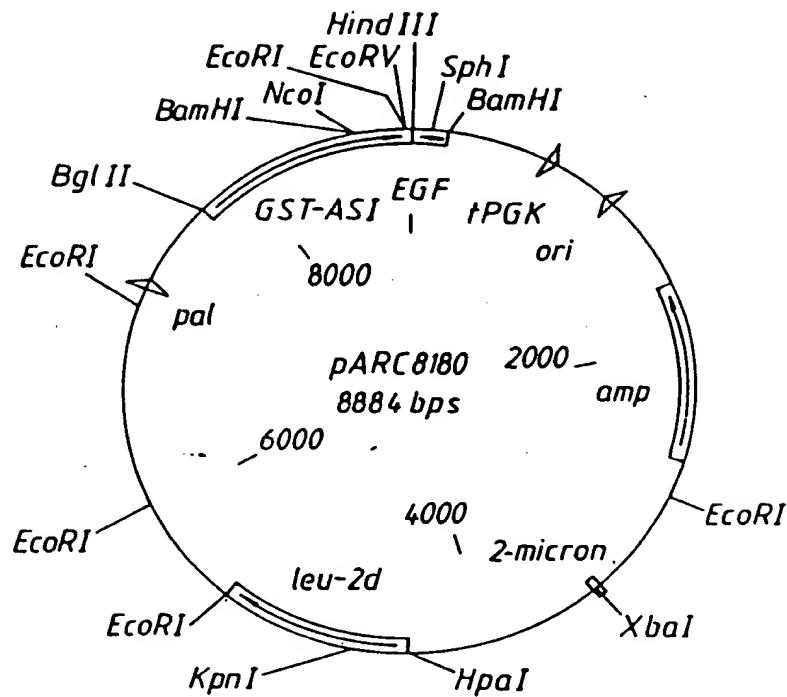
F-9.5

(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.



F-79-6

Amratlon
(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.



F-79-7

Acralion

(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

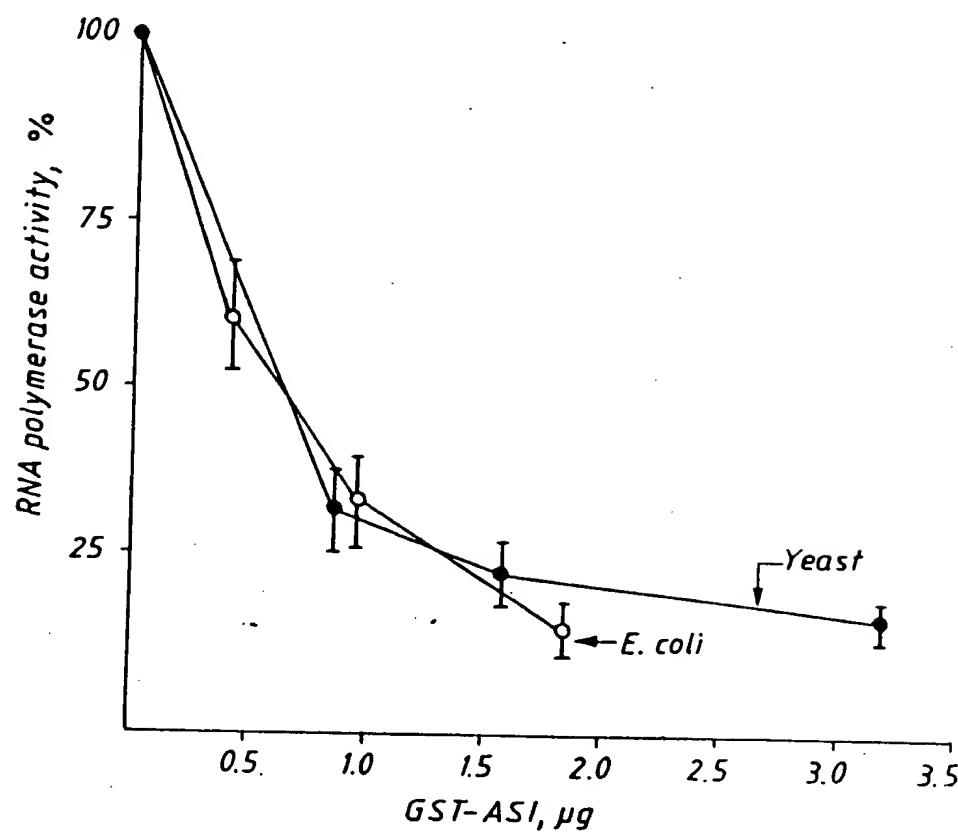


Fig. 8

Acquisition

(MRS. A.V. NATHAN)
AGENT FOR THE APPLICANT.

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